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Inhibition of Calcium-dependent Protein Kinase C by Hexadecylphosphocholine and 1-*O*-Octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine do not Correlate With Inhibition of Proliferation of HL60 and K562 Cell Lines

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We investigated the hypothesis that the antiproliferative effect of hexadecylphosphocholine (HePC) and 1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine (ET-18-OCH_3) is mediated through the inhibition of cellular protein kinase C (PKC). In the sensitive HL60 cell line, ID_{50} and LD_{50} values of 5.6 and 5.3 μM, respectively (HePC), and of 3.8 and 4.2 μM, respectively (ET-18-OCH_3) were obtained. In the more resistant K562 cell line, these values were 69.1 and > 97 μM, respectively (HePC) and 7.8 and 76.8 μM, respectively (ET-18-OCH_3). Treatment of both cell lines with HePC and ET-18-OCH_3 (25 μM) for 2 h did not lead to PKC translocation. However, a 30% reduction of PKC activity, mainly due to a decrease in the cytosolic compartment, was found. Half maximal stimulation of PKC translocation by phorbolester (TPA) in HL60 and K562 cells, which were pretreated for 2 h with 25 μM of the lipids, resulted in a 20–30% decrease of membrane-bound PKC, whereas the cytosolic form was found to be unchanged. In the same experimental setting, dioctanoylglycerol (DIC_8)-stimulated PKC translocation was not affected by HePC or ET-18-OCH_3. However, a 10–20% reduction of PKC enzyme activity in the membrane and in the cytosolic fraction was obtained. These findings indicate that HePC and ET-18-OCH_3 do not interfere with PKC translocation but rather mediate a general decrease of the enzyme activity in the membrane and cytosol of the cells. Since the extent of PKC inhibition was somewhat similar in the sensitive HL60 and the resistant K562 cell line, inhibition of PKC is probably not a prerequisite for the antiproliferative action of HePC and ET-18-OCH_3.

Key words: hexadecylphosphocholine, alkylphosphocholines, ET-18-OCH_3, etherphospholipids, protein kinase C

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INTRODUCTION

HEXADECYLPHOSPHOCHOLINE (HePC) and 1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine (ET-18-OCH_3) represent a new class of phospholipid-derived, membrane active antineoplastic agents. ET-18-OCH_3 is the prototype of the cytotoxic etherlysolipids. The alkylphosphocholine HePC was developed in studies on structure–activity relationship of alkyllysophospholipids, and possibly represents the minimal structural requirement of these compounds [1]. HePC displays distinct cytotoxic effects towards a broad variety of tumour cell lines *in vitro* and some animal tumours *in vivo* [2–6].

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Up to now, the molecular mechanism by which HePC and etherlipids exert their biological activities is still unknown. So far, several possible target structures have been proposed. ET-18-OCH_3 and HePC have been described to interfere with the membrane phospholipid synthesis. In particular, these compounds inhibit the incorporation of fatty acids and choline into phosphatidylcholine [7–11]. Recently, some evidence has been provided that ET-18-OCH_3 and HePC may block an important cellular signal transduction pathway by inhibiting phosphatidylinositol-specific phospholipase C [12,13]. Two other enzymes affected by HePC and ET-18-OCH_3 seem to be the Na^+, K^+-ATPase in HL60 cells [14] and the Na^+, H^+-antiporter in NIH 3T3 cells [13].

One crucial event for intracellular signal transduction of many hormones and growth factors is the activation of protein kinase C (PKC) (reviewed by Nishizuka [15,16]). It is now well established that alkyllysophospholipids, like ET-18-OCH_3 and analogues, possess PKC inhibitory activity [11,17–20]. Recently it was shown that HePC also inhibits PKC in cell free systems

[13, 21]. However, it has not yet been reported whether HePC or ET-18-OCH_3 could inhibit PKC activation in intact cells and, furthermore, whether PKC inhibition is a necessary prerequisite for the cytotoxic effect of these compounds.

In this study, we investigated the influence of HePC and ET-18-OCH_3 on PKC activity, and on phorbolester (TPA) and dioctanoylglycerol (DIC_8)-induced PKC translocation in HL60 and K562 human leukaemia cell lines. Since the HL60 cell line is very sensitive against etherlipids and analogues, but K562 cells are not, we examined the significance of PKC inhibition with respect to the antiproliferative activity of both compounds towards HL60 and K562 cells.

MATERIALS AND METHODS

Cell culture

Cell lines were purchased from the American Type Culture Collection (Rockville, U.S.A.) and maintained in Click's/RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 10 mM glutamine, 10000 U/ml penicillin, 120 U/ml streptomycin and 20 mM HEPES pH 7.3. All cell culture material was from Gibco (Glasgow, Scotland).

Materials

HePC and ET-18-OCH_3 were synthesized in our laboratory as previously described [3]. Bovine serum albumin (BSA), histone III-S, aprotinin, leupeptin, TPA, ionomycin, phosphatidylserine and diolein, 1,2-dioctanoylglycerol were from Sigma (Munich, Germany), phenylmethane sulphonyl fluoride (PMSF), ethyleneglycoltetraacetic acid (EGTA) and EDTA were from Merck (Darmstadt, Germany). [^{3}H]Thymidine (70–90 μCi/mmol) was purchased from Amersham (Braunschweig, Germany) and [^{32}P]ATP (30 Ci/mmol) from DuPont/NEN (Dreieich, Germany).

Cell proliferation assays

Two million cells were incubated in 5 ml medium in Nunc 6-well plates (Wiesbaden, Germany) with escalating doses of either HePC or ET-18-OCH_3 at 37°C for 48 h. For the last 12 h of incubation, 10 μCi/well [^{3}H]thymidine were added. Aliquots of the cell suspension were taken, transferred to and washed on Whatman glassfibre filters with a PHD cell harvester (Cambridge, U.S.A.). Filters were dried and cell incorporated radioactivity was quantified in a Packard 1900 CA liquid scintillation analyser (Frankfurt, Germany). Cell number was counted in a Neubaur chamber, cell viability was determined by the trypan blue dye exclusion assay.

PKC translocation and preparation

Twenty million cells/5 ml medium were incubated in a water bath at 37°C for different periods of time with 25 μM HePC or ET-18-OCH_3. During incubation, the proteinase inhibitors PMSF (0.1 mM), aprotinin (10 μg/ml) and leupeptin (100 μg/ml) were present in the medium. At indicated time intervals, PKC translocation was induced by addition of 10 ng/ml TPA for 10 min or 10 μg/ml DIC_8 for 5 min. These concentrations and incubation times were chosen in order to achieve a half maximal translocation of PKC from cytosol to the membrane. Incubations were stopped by addition of ice-cold PBS and immersion of tubes in a methanol bath at −20°C for 15 s. Cells were washed twice in cold PBS and resuspended in homogenisation buffer containing 20 mM Tris pH 7.5, 2 mM EDTA, 5 mM dithiothreitol, 0.1 mM PMSF, 100 μg/ml leupeptin and 10 μg/ml aprotinin.

Cells were homogenised at 4°C by 30 strokes at 1000 rpm in a Potter-Elvehjem homogeniser. Cell disruption was checked microscopically. Nuclei were sedimented at 100 *g* for 5 min. In a second centrifugation step at 100000 *g* for 60 min, membranes were separated from cytosol. The membrane pellets were further processed by elution of proteins from the lipid matrix by 0.1% Triton for 60 min and a third centrifugation step at 100000 *g* for 5 min at 4°C. Protein content of each fraction was determined according to the method of Lowry, modified by Peterson [22].

Phosphorylation assay

PKC activity was determined by phosphorylation of its specific substrate histone III-S in the presence of [^{32}P]ATP.

Separation of PKC from other kinases was achieved by passing the subcellular fractions through DEAE-cellulose columns [23]. PKC was eluted with 1 ml 100 mM NaCl, and stabilised with 1 mg/ml BSA. Phosphorylation assays were performed with 50 μg of protein from each fraction in 100 μM reaction buffer containing 20 mM HEPES pH 7.4, 10 mM magnesium chloride, 0.5 mM calcium chloride, 0.25 mg/ml histone III-S, 50 μg/ml phosphatidylserine and 5 μg diolein. Reactions were started by addition of 50 μM [^{32}P]ATP (40 nCi/nmol) at 30°C and terminated after 10 min with ice-cold stop buffer consisting of 50 mM potassium phosphate pH 7.0, 10 mM EGTA, 10 mM EDTA, 5 mM ATP and 5 mg/ml BSA. Proteins were precipitated with 12.5% trichloroacetic acid (TCA) on Whatman glassfibre filters. Filter-bound radioactivity was measured in a Packard 1900 CA liquid scintillation counter.

Competition assays

PKC was prepared from the cytosolic fraction of untreated HL60 cells as described above. Fifty micrograms of protein were employed in a phosphorylation assay in the presence of 25 μM HePC. The reaction buffer was modified by various concentrations of either phosphatidylserine, diolein or calcium.

PKC activity in all phosphorylation assays was expressed as a percentage of total PKC activity in membrane and cytosolic fractions of untreated control cultures. Non-specific histone phosphorylation in the absence of phosphatidylserine and diolein was subtracted.

RESULTS

Table 1 summarises results from cell proliferation assays after 48 h of treatment with various concentrations of HePC and ET-18-OCH_3. In HL60 cells, the concentrations of HePC and ET-18-OCH_3 necessary to achieve a half maximal cytostatic (ID_{50}) or cytotoxic (LD_{50}) effect did not differ significantly for each

Table 1. Sensitivity of HL60 and K562 cells towards HePC and ET-18-OCH_3

Substances	Cell line	ID_{50}*(μM)	ID_{50}†(μM)	LD_{50}‡(μM)
HePC				
	HL60	5.6 ± 1.5	6.8 ± 1.4	5.3 ± 0.9
	K562	69.1 ± 14.4	58.9 ± 6.6	> 97
ET-18-OCH_3				
	HL60	3.8 ± 0.5	4.4 ± 0.9	4.2 ± 0.7
	K562	7.8 ± 2.6	7.2 ± 1.5	76.8 ± 11.6

ID_{50} values were calculated from *[^{3}H]thymidine incorporation data or †cell count. ‡LD_{50} values were calculated from trypan blue dye exclusion assays. Data are the mean values ± S.D. of six independent experiments.

compound. Both substances displayed similar antiproliferative activity, with ET-18-OCH_3 being the slightly more potent drug in this sensitive cell line. In K562 cells, ID_{50} concentrations for HePC were approximately 10-fold higher, and for ET-18-OCH_3 almost twice as high as in HL60 cells. LD_{50} values for both compounds were above 70 μM. This indicates that the K562 cell line is somewhat resistant against these lipids.

For all further experiments with PKC, we chose a constant concentration of both compounds of 25 μM for several reasons. First, HePC and ET-18-OCH_3 enter cells by a endocytotic mechanism [24, 25]. It is, therefore, possible to load cells with equal amounts of compound in a shorter incubation time at higher concentrations in the medium. Second, HePC uptake kinetics in HL60 and K562 cells are almost identical. Under these conditions, both cell lines show HePC membrane concentrations of 2 nmol per 100 nmol cellular phospholipid after 2 h of exposure [26]. At this short incubation time most of the compound should be located in the plasma membrane. Third, non-specific toxicity seems very unlikely as cell viability does not differ from control cell cultures and the minimal micellar concentration for HePC and ET-18-OCH_3 is far above 140 μM [27].

To characterise the mode of HePC action on PKC in kinetic studies, we employed partially purified PKC from HL60 cells in a standardised histone III-S phosphorylation assay (Figure 1). At constant drug concentrations (25 μM), HePC produced an inhibitory effect on PKC which could be overcome by increasing amounts of phosphatidylserine but not by diacylglycerol (data not shown). Lineweaver–Burk plot analysis revealed a competitive type of antagonism with respect to phosphatidylserine with a K_i of 25.1 μM. These data confirmed similar results reported previously for HePC [13], ET-18-OCH_3 [14] and the thioetherlipid BM 41.440 [17]. To investigate the influence of HePC and ET-18-OCH_3 on PKC in intact cells, we measured the effects of both compounds on basal PKC activity in cytosol and membranes (Figure 2).

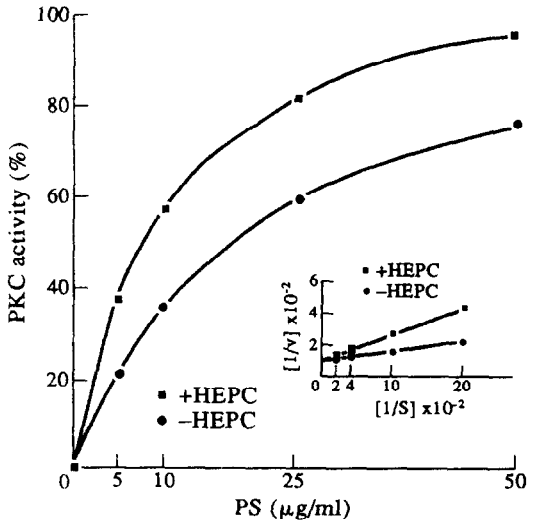

Figure 1. Inhibition of PKC by HePC as a function of phosphatidylserine concentrations. Total PKC activity from HL60 cells was assayed by histone III-S phosphorylation with (+) or without (−) 25 μM HePC, 5 μg/ml diolein and increasing amounts of phosphatidylserine. Non-specific activity in the absence of lipids was subtracted. Each point represents the mean of three independent experiments with S.D. values of less than 10%. The insert shows the double reciprocal plot of these data.

Treatment of HL60 and K562 cells with 25 μM of either compound did not lead to a translocation of PKC. On the contrary, a 30% decrease of total PKC activity after 2 h of incubation was obtained. Analysis of membrane- and cytosol-associated PKC revealed that the reduction of total enzyme activity was mainly due to a decrease of the cytosolic form. These observations are in good agreement with results reported by Überall and colleagues showing that HePC inhibits the PKC-mediated activation of the Na^+/H^+ antiporter in intact NIH 3T3 cells [13].

In order to assess the effects of HePC and ET-18-OCH_3 on PKC translocation the cells were incubated with 25 μM of these substances and then stimulated either by TPA or DIC_8 in concentrations chosen to achieve half maximal PKC translocation (Figure 3).

In TPA-stimulated cells, both compounds reduced membrane-bound PKC activity by 20–30%. Cytosolic PKC levels remained unchanged. This is in contrast with the results in unstimulated cells where a decrease of enzyme activity was only detected in the cytosol. When PKC translocation was stimulated with the more physiological PKC activator analogue DIC_8 in the same experimental setting, HePC and ET-18-OCH_3 induced a reduction of PKC activity in both the cytosolic and the membrane preparation (Figure 4).

DISCUSSION

To investigate whether PKC is involved in the antiproliferative action of HePc and ET-18-OCH_3, the effect of both compounds on cell proliferation and on PKC activity was studied. Experiments were carried out on HL60 and K562 human leukaemic cell lines, since they have been shown to possess different *in vitro* susceptibility towards HePC and ET-18-OCH_3 [10, 19].

Our findings suggest that HePC and ET-18-OCH_3 do not interfere with calcium-dependent translocation of PKC but rather mediate a general decrease of enzyme activity in cytosol and membrane, possibly explained by a competitive inhibition with regard to phosphatidylserine. Supporting this, Salari and colleagues described reduced levels of PKC activity in cell membranes of WEHI-3B cells pretreated with the etherlipid ET-16-OCH_3-GPC while [^{3}H]PDBU binding to the same membrane preparation was unimpaired [28]. This indicates that the amount of enzyme does not change while the biological activity decreases. Similar results were also obtained with the thioetherlipid BM 41.440 in the human leukaemia cell lines HL60 and KG-1 [17].

However, these results have to be interpreted cautiously, because so far all investigations on PKC inhibition by etherlipids have been performed under conditions suitable for testing calcium-dependent PKC isoforms. Therefore, an inhibitory action of ET-18-OCH_3 and HePC can only be applied to the calcium-dependent PKC isoforms (PKC-α, -β I and -β II). Currently, it is not known whether calcium-independent or phorbolester-unresponsive PKC isoforms might be affected differently. This fact is especially important because a change of PKC isoform pattern during neutrophil differentiation in HL60 cells [29, 30] may be a mechanism by which these cells become resistant towards the toxic effects of etherlipids [31]. Alternatively, some biological effects of etherlipids could be mediated via calcium-independent PKC isoforms.

Two other aspects of etherlipid-induced inhibition of PKC need to be considered and discussed in more detail. Firstly, though etherlipids are incorporated in cellular membranes, a decrease of cytosolic PKC activity was apparent in addition to an inhibition of membrane-bound PKC. This can probably be

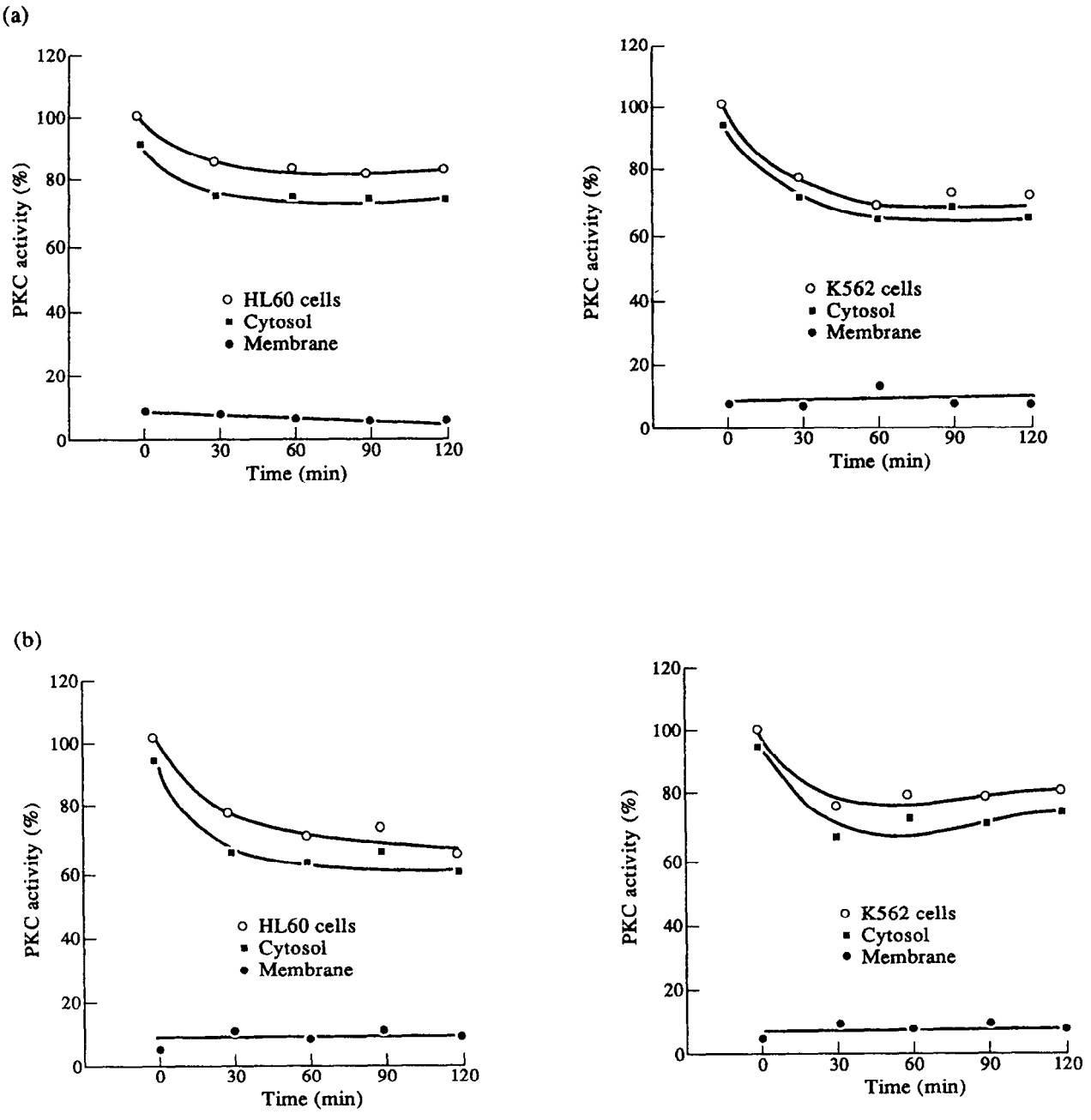

Figure 2. Effects of 25 μM HePC (a) and ET-18-OCH_3 (b) on PKC activity in intact HL60 and K562 cells. Twenty million cells were incubated with HePC or ET-18-OCH_3 for indicated time periods. Specific PKC activity was determined by histone III-S phosphorylation and subtraction of unspecific phospholipid-independent activity in absence of phosphatidylserine and diolein. Data represent the mean of total (●), membrane bound (◆) and cytosolic PKC activity (■) of three independent experiments expressed as % of untreated control culture. S.D. was always less than 10%.

explained by contact of PKC with intracellular vesicles, which were generated at the plasma membrane during endocytosis, containing sufficient amounts of etherlipid [25].

Secondly, some doubts about the competitive character of PKC inhibition by etherlipids were raised by Hesbeen and colleagues, who found even increased PKC activity when ET-18-OCH_3, PS and diolein were added as mixed liposomes into the assay [32]. These authors also saw an activation of PKC when membrane-associated enzyme was assayed in its natural phospholipid environment plus ET-18-OCH_3 [32]. This work clearly shows how PKC activity changes, induced by etherlipids, may very much depend on the physical state of the lipid environment in different assay systems. A precise definition of assay conditions in all further comparative investigations on the effects of etherlipid on PKC seems extremely important. We could not confirm this activation of PKC in our experimental setting. The fact, however, that we measured less PKC activity after the enzyme had been exposed to etherlipids for a short time and then extracted from its lipid environment, seems to reflect an irreversible effect of the etherlipids on PKC than a competitive enzyme inhibition.

According to our observations, it does not appear likely that the antiproliferative action of HePC and ET-18-OCH_3 is mediated by inhibition of calcium-dependent PKC. Despite being resistant towards the cytotoxic effects of HePC and ET-18-OCH_3, PKC activity reduction was equally pronounced in K562 cells as in the sensitive cell line HL60.

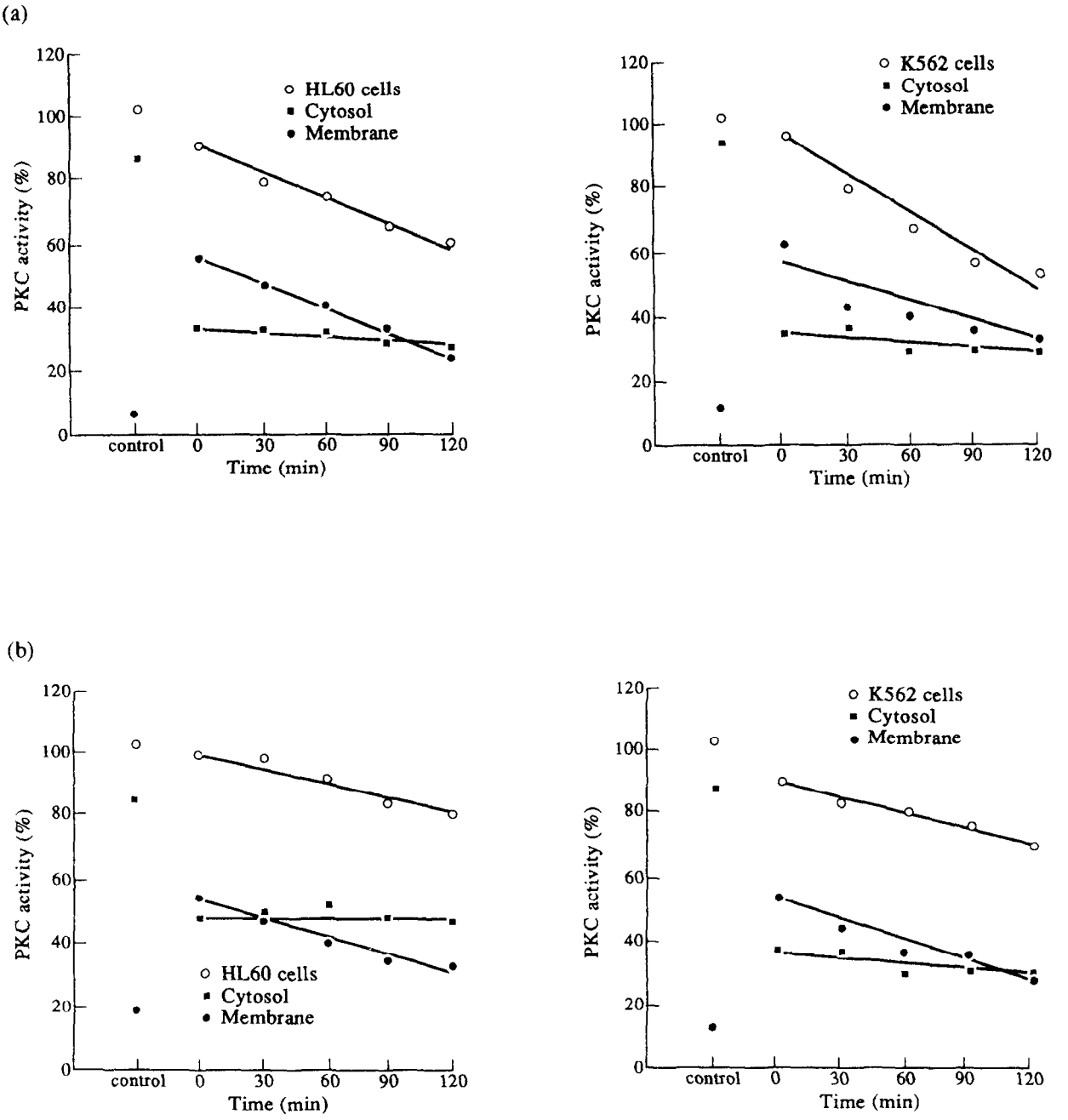

Figure 3. Effects of 25 μM HePC (a) and ET-18-OCH_3 (b) on TPA-induced PKC translocation in HL60 and K562 cells. Twenty million cells were incubated with HePC or ET-18-OCH_3 for indicated time periods. PKC translocation was stimulated by 10 ng/ml TPA for 10 min. Specific PKC activity was determined by histone III-S phosphorylation and subtraction of non-specific phosphorylation in the absence of phosphatidylserine and diolein. Data show the mean of total (●), membrane bound (◆) and cytosolic PKC activity (■) of three independent experiments expressed as % of untreated control. S.D. was always less than 10%.

With respect to ET-18-OCH_3, one could argue that PKC inhibition at least correlates with the cytostatic properties of this compound in both cell lines. But then ET-18-OCH_3 at the same molar concentration should inhibit PKC in HL60 cells twice as effective as in K562 cells.

Treatment of HL60 and U937 cells with 25 μM HePC for 1 h leads to a substantial reduction of c-*myc* mRNA levels [33]. In U937 cells, HePC and ET-18-OCH_3 induce histone HI° mRNA formation after 1–3 h of incubation, followed by growth arrest and expression of differentiation markers on the cell membrane [34]. PKC activity, however, is reduced only by 20–30% during this time period. Though a further decrease of PKC activity up to 40% compared to control values can be achieved by prolongation of HePC treatment for 24 h, the signals which mediate the antiproliferative effects of HePC and ET-18-OCH_3 apparently function on a different time scale. By using another approach, a similar conclusion is drawn by Salari and colleagues [28]. These authors used the etherlipid sensitive cell line WEHI-3B and the resistant cell line R6X-B15, and treated them with two different etherlipids, one with potent cytotoxic activity (ET-16-OCH_3-GPC) and the other with none (ET-16-OCH_3-thio-Glc). They showed that these compounds inhibited TPA-induced PKC activity in the membrane fraction by 20–30% in both cell lines, independently of their cytotoxic potential.

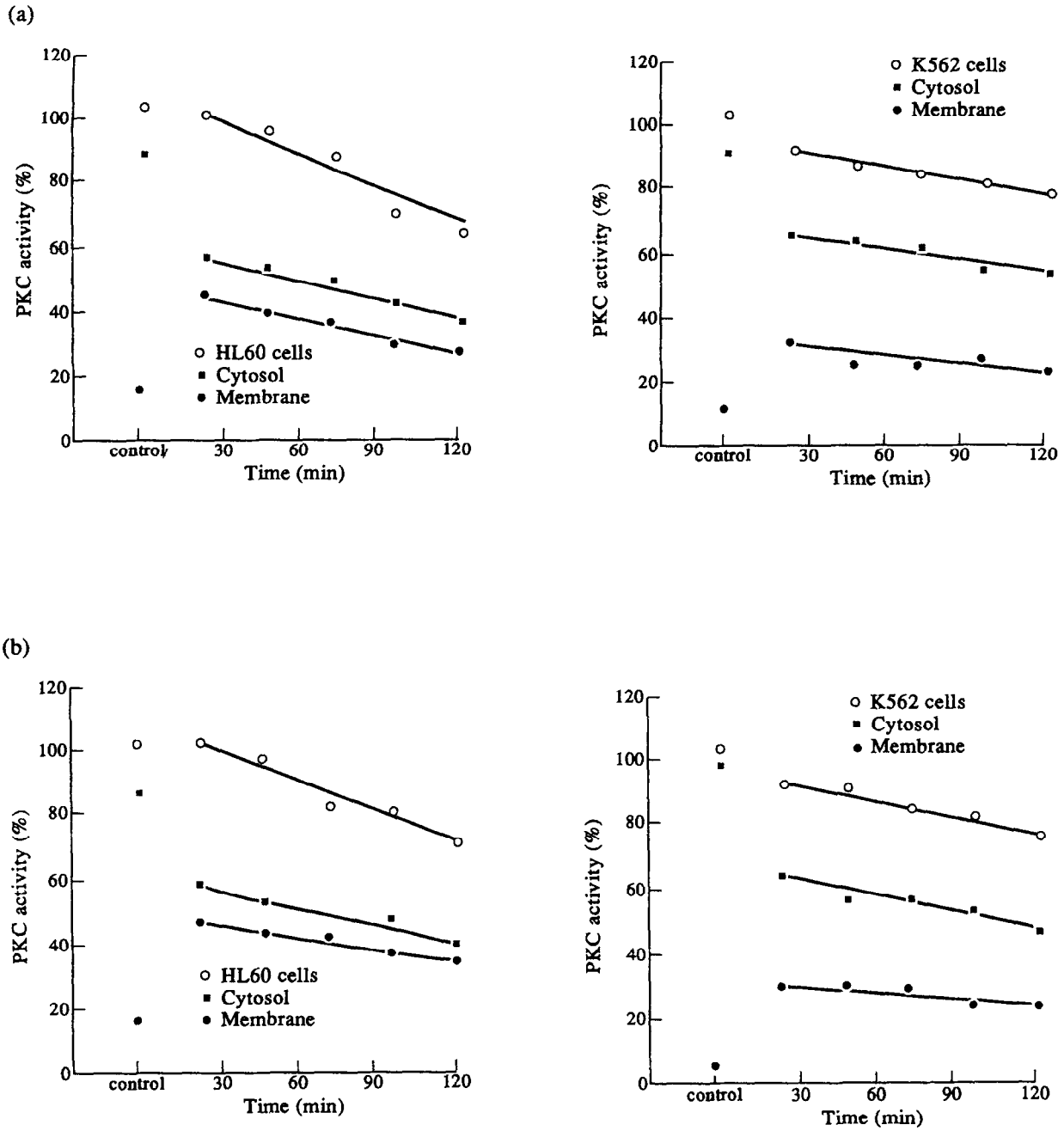

Figure 4. Effects of 25 μM HePC (a) and ET-18-OCH_3 (b) on DIC_8-induced PKC translocation in HL60 and K562 cells. Twenty million cells were incubated with HePC or ET-18-OCH_3 for indicated time periods. PKC translocation was induced by 10 μg/ml DIC_8 in the presence of 10 μg/ml ionomycin for 5 min. Specific PKC activity was determined by histone III-S phosphorylation and subtraction of phospholipid-independent activity in the absence of phosphatidylserine and diolein. Data represent the mean of total (●), membrane bound (◆) and cytosolic PKC activity (■) of three independent experiments expressed as % of untreated control culture. S.D. was always less than 10%.

HePC and etherlipids like ET-18-OCH_3 are considered to be potent PKC inhibitors and antagonists of some PKC-mediated biological effects, like induction of differentiation and, consecutively, growth inhibition. However, the results of this communication and the work of Salari and colleagues raise serious doubts on the possible involvement of calcium-dependent PKC in the antitumoural action of HePC and ET-18-OCH_3.

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